Identifying and Quantifying *Phakopsora pachyrhizi* Spores in Rain

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ABSTRACT

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In summers of 2005 and 2006, rain was collected weekly at over 100 selected National Atmospheric Deposition Program/National Trends Network sites across the soybean-growing region of the central and eastern United States. Rain samples were screened for *Phakopsora pachyrhizi* (causal agent of soybean rust) DNA using a nested real-time polymerase chain reaction assay. Over this time frame, *P. pachyrhizi* spores were detected in every state in the study, but more frequently in states along the Gulf and Atlantic coasts and along the Ohio River Valley westward to Kansas. A bimodal temporal distribution of samples testing positive for *P. pachyrhizi* was found in both years. However, there was a greater than threefold increase in the number of samples testing positive for *P. pachyrhizi* in 2006 compared with 2005, with the most significant increase in August. There was also an increase in the average number of spores per sample in 2006 relative to 2005. Sequence analysis of a subset of positive samples was used to validate the assay results. From the sequence analysis, two reliable polymorphic regions were found, resulting in six distinct genotypes. One genotype was found in 56% of the samples tested, whereas the other genotypes were found less frequently.

Additional keywords: long-distance dispersal (LDD), Pest Information Platform for Extension and Education (PIPE).

Soybean rust (SBR) caused by *Phakopsora pachyrhizi* Syd. & P. Syd. can be a devastating disease of soybean, with losses reported in Asia as high as 80% (4). For most of the twentieth century, however, SBR was generally restricted to Asia, until the disease began to rapidly spread in the 1990s. The disease was first reported in Hawaii in 1994 (12), Africa in 1996 (14), South America in 2001 (17), and the continental United States in 2004 (26). SBR is now a concern in all major soybean-growing regions of the world.

Conditions favorable for SBR development do occur in a typical growing season throughout most of the major U.S. soybean production area (3,15) but not year-round (20). Because no aecial host is known (3), overwintering, or survival between periods of host availability, is believed to be restricted to urediniospore survival, which is restricted by temperature, moisture, and available living host tissue (15,20). Within the continental United States, those areas are largely confined to perennial weed legumes in the southern parts of states bordering the Gulf of Mexico (15,19). Additional inocula sources could be from infected legume hosts in Mexico and islands of the Caribbean. Therefore, long-distance dispersal of airborne urediniospores would be necessary if SBR were to become a problem for the major soybean production area of the central United States.

Long-distance dispersal (LDD) of fungal spores that are ultimately deposited in rain showers has been observed in North America (23,25,28), in Europe (9,10), and intercontinentally (5,18,22). The role of rain-deposited urediniospores in establishing primary infection by *Puccinia graminis* and *P. triticina* on wheat in the northern wheat-growing areas of the United States has been shown (25). In comparing different types of spore traps, Roelfs et al. (23) recovered stem rust urediniospores in rain samples ≈10 days before trapping them on 5-mm rod impaction traps. In their study, detection of rust spores on impaction traps generally coincided with the local spread of spores within and between fields. Therefore, at least for ground-based detection systems, the use of rain traps to detect the initial arrival of spores prior to disease development is most useful.

Past studies of spore transport in rain have generally utilized only a small number of traps, making spread of primary inoculum into a broad geographic region difficult to detect. Therefore, to monitor dispersal of spores from a recently introduced pathogen, such as *Phakopsora pachyrhizi* where inocula sources are relatively unknown or not well established, deploying a greater number of traps at a larger scale would be ideal. The National Atmospheric Deposition Program/National Trends Network (NADP/NTN; available online) provides a nationwide network of monitoring sites where precipitation is collected weekly. Established in 1978 with 22 sites, it currently exceeds 250 sites, spanning the continental United States, Alaska, Puerto Rico, and the Virgin Islands.

Information on *P. pachyrhizi* urediniospore transport into soybean-growing regions of the United States where the rust fungus does not normally overwinter would be useful to predict epidemics or to guide scouting efforts. The objectives of this study were (i) to develop an assay sensitive enough to detect one or more spores suitable for monitoring the initial deposition of inoculum and (ii) to assay precipitation collected at NADP/NTN stations located across the major soybean-growing regions of the continental United States to assess patterns of spore deposition during the soybean-growing season.

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains two tables showing data for each site in 2005 and 2006 and a figure showing DNA sequence alignment for the complete set of samples.

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MATERIALS AND METHODS

Laboratory precautions. One general concern in this study was the risk of contamination when using a nested real-time quantitative polymerase chain reaction (qPCR) method to detect DNA. Therefore, precautions were followed regarding the laboratory equipment and practice. Separate working rooms or areas were used for the preparation of positive controls, rain filter preparation, DNA extraction, and qPCR. A set of micropipettes was designated to be used solely to prepare the mastermix for the assays, and never came in contact with DNA. In filter preparation, disposable laboratory gloves and razor blades were changed between each filter. All assay reagents were aliquoted in a BioSafety cabinet (SterilGard; The Baker Company, Sanford, ME), frozen and used once, and discarded each day. The assay reagents aliquoted included primers, the TaqMan probe, purchased sterile water, Tris EDTA (TE), and Tris buffers. OmniMix (Packaged for TaKaRa Bio Inc. by Cepheid, Sunnyvale, CA), used as the mastermix, is in a bead form with one bead per tube. For each set of assay reactions, samples and the negative control were always prepared before the positive control.

Contamination, estimated by the number of negative controls with a positive threshold cycle (C_T) value, occurred in 4% of the assays in 2005 and 6% of the assays in 2006. The frequency of contamination increased when the number of rain samples testing positive increased in both 2005 and 2006, and decreased when the frequency of positives samples fell (data not shown). When the negative control of an assay showed a positive result, all assay reagents were discarded, new aliquots were used, and the assay was rerun. If the negative control still showed a positive result, all samples in that assay were considered contaminated. Using new reagent aliquots worked to eliminate the false positive occasionally, reducing the number of samples being lost to contamination to 3% in 2005 and roughly 4% in 2006.

Rain sample collection and sample preparation. Each week, precipitation from individual NADP/NTN sites was sent to the Illinois State Water Survey in Champaign, IL, and filtered according to a standard protocol with a minimum volume of 0.02 in. (7). Samples were filtered in total or for 1 h if filtration was slow. Filters were dried for 24 h in individual petri dishes. Once dried, petri dishes were sealed and sent overnight to the Cereal Disease Laboratory (CDL) in St. Paul, MN. Filters were assayed from rain collected at 121 and 110 selected NADP/NTN sites across the soybean-growing region of the central and eastern United States in 2005 and 2006, respectively (Fig. 1). Rain was assayed from weekly collections made between 10 May and 30 August in 2005 and from 9 May to 17 October 2006. At the CDL, rain filters were handled under sterile conditions in a biosafety cabinet. Filters were cut in half with a disposable razor blade, with one-half of the filter assayed for *P. pachyrhizi* and the other stored at –80°C. Each half filter to be assayed was fan-folded with a fresh pair of latex gloves and placed in a 2-ml microcentrifuge tube. Filters were submersed in 10 mM Tris-HCl buffer (pH 8.0), soaked in a 65°C water bath for 5 min, and sonicated for 1 min. Filters were inverted with sterile toothpicks and soaked and sonicated a second time as above. Filters were removed with a sterile toothpick, placed in a new 1.5-ml microcentrifuge tube, and spun briefly to remove residual buffer. Samples were dried by centrifugation in a Speed Vac SC110 (Thermo Fisher Scientific, Inc., Waltham, MA) overnight. A separate 8.0-µm filter was placed over the air intake opening to prevent movement of spores into and out of the Speed Vac. The following morning, the dried samples were shaken with 10–15 1-mm glass beads in a Savant FastPrep shaker (FP120; Holbrook, NY) for 20 s at a speed setting of 4, spun down, and shaken a second time at the same setting. DNA was extracted using the OmniPrep Genomic DNA kit (GenoTech Inc., St. Louis) and using muscle glycogen as a DNA carrier. DNA was stored dry at –20°C until assayed. To assay samples for *P. pachyrhizi*, samples were rehydrated overnight in 15 µl of sterile water. Assays were performed immediately the following morning. TE (2 µl) was added to the remaining sample after it was assayed and stored at –80°C.

Assay development. A primary goal of this study was to develop an assay to consistently detect a single spore of *P. pachyrhizi* in rain. Three assays were evaluated. The first assay was adapted from Barnes and Szabo (2) using the ITS1rustF10d forward primer with a reverse primer ITS1rustR3d. The reverse primer was modified from a similar primer (ITS1rustR3c) (2) to move a single nucleotide mismatch toward the 5′ end of the primer. A TaqMan probe was designed specifically for *P. pachy-*

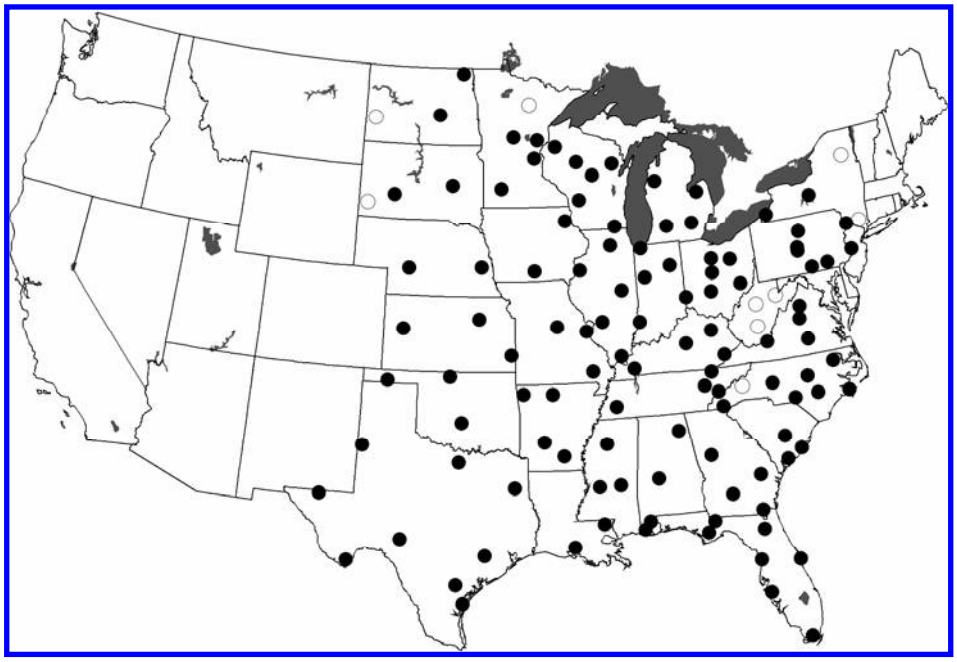

Fig. 1. National Atmospheric Deposition Program/National Trends Network sites monitored for *Phakopsora pachyrhizi* in this study. Solid circles were monitored in 2005 and 2006; sites with open circles were monitored in 2005 only. One site in Puerto Rico and one site in the Virgin Islands (not shown) were also monitored in 2005 only.

rhizi in the internal transcribed spacer 1 (ITS1) region. The *P. pachyrhizi*-specific probe ITS1PhpFAM1 (5′-FAM-TCATTGATTGATAAGATCTTTGGGCAATGG-3IABlkFQ) (Integrated DNA Technologies Coralville, IA) and reverse primer ITS1rustR3d (5′-TGTGAGAGCCTAGAGATCCATTG) were designed by comparing sequences of the ribosomal DNA (rDNA) for rust fungal species listed in Table 1 using MacVector (MacVector, Inc., Cary, NC). The assay was performed in a 25-µl final volume with 1× OmniMix (TaKaRa Bio Inc. by Cepheid), 0.5 µM each primer, 0.2 µM ITS1PhpFAM1, and 2 µl of sample DNA. Cycling parameters were 95°C for 2 min, followed by 45 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s.

The second assay evaluated was the assay developed by Frederick et al. (8). Assay conditions were followed as described.

The third assay was a nested assay combining the first two assays. The nested assay used the reverse primer Ppa2 specific to *P. pachyrhizi* (8) and a more general rust fungal forward primer ITS1rustF4a (5′-GAGGAAGTAAAAGTCGTAACAAGGTTTC) in the first round. The first-round amplification was performed in a final volume of 25 µl containing 1× OmniMix, 0.5 µM each primer, and 2 µl of sample DNA. Cycling parameters were 95°C for 2 min, followed by 20 amplification cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The amplicon was diluted 1:1000 in sterile water in two steps, 10:90 and 10:990, and immediately used as template for the second-round PCR. Cycling parameters for the second-round qPCR were identical to the conditions of the first assay but used 2 µl of the diluted amplicon as the DNA template. All three assays were performed on a SmartCycler (Cepheid). Fluorescence data calculated by the SmartCycler software (ver. 2.0c) were collected at the end of each 60°C annealing step.

Controls for final assay. Each assay contained 15 rain samples, a positive control, and at least one negative control. The sources of *P. pachyrhizi* DNA for the positive control were different in 2005 and 2006 but were consistent throughout a year. In 2005, *P. pachyrhizi* DNA was obtained from spores collected from infected soybean leaves at the BL-3 facility at Fort Detrick, MD (access to infected plants courtesy of Dr. Reid Frederick, United States Department of Agriculture–Agricultural Research Service, Foreign Disease–Weed Science Research Unit, Fort Detrick, MD). Only DNA was removed from the facility and was shipped with APHIS permits to the CDL in St. Paul, MN for use in this study. In 2006, permits to work on infected tissue were obtained. *P. pachyrhizi* DNA was extracted from 20 individual infected soybean plants collected on 18 August 2005 from a sentinel plot in Marion County, FL by Dr. Carrie Lapaire Harmon (University of Florida, Gainesville) and sent to the CDL the same day. Dilutions of a single DNA sample were used for all assays in 2006.

For each set of 15 NADP/NTN filters, a blank filter was added during the filter-cutting phase as a control for contamination between cutting filters. When a NADP/NTN site either did not have precipitation over a weekly interval or precipitation was too

TABLE 1. List of rust fungal samples used to design and test fungal and rust specific primers and *P. pachyrhizi* specific TaqMan probe

Rust species	Host	Origin	Source[a]	Year	GenBank accession no.
Phakopsora pachyrhizi					
Australia 72-1[b]	*Glycine max*	Australia	6	1972	AF333488
Australia 79-1[b]	*G. max*	Australia	6	1979	AF333489
Hawaii 95[b]	*G. max*	Hawaii	6	1995	AF333490
Hawaii 98[b]	*G. max*	Hawaii	6	1998	AF333491
India 73-1[b]	*G. max*	India	6	1973	AF333492
Indonesia 72-1[b]	*G. max*	Indonesia	6	1972	AF333493
Philippines 77-1[b]	*G. max*	Philippines	6	1977	AF333494
Taiwan 72-1[b]	*G. max*	Taiwan	6	1972	AF333495
Taiwan 80-1[b]	*G. max*	Taiwan	6	1980	AF333496
Taiwan 80-2[b]	*G. max*	Taiwan	6	1980	AF333497
Thailand 76-1[b]	*G. max*	Thailand	6	1976	AF333498
MUT Zimbabwe[b]	*G. max*	Zimbabwe	6	2000	AF333499
TM Zimbabwe[b]	*G. max*	Zimbabwe	6	2000	AF333500
Php1[c]	*G. max*	NA	6	2005	…
HSZ1685-A5	*G. max*	Florida	7	2005	EU436700
HSZ1685-T5	*G. max*	Florida	7	2005	EU436701
HSZ1686-T5	*G. max*	Florida	7	2005	EU436702
HSZ1687-A5	*G. max*	Florida	7	2005	EU436703
HSZ1687-T5	*G. max*	Florida	7	2005	EU436704
HSZ1688-T5	*G. max*	Florida	7	2005	EU436705
HSZ1689-A5	*G. max*	Florida	7	2005	EU436706
HSZ1690-A5	*G. max*	Florida	7	2005	EU436707
HSZ1690-T5	*G. max*	Florida	7	2005	EU436708
HSZ1691-T5	*G. max*	Florida	7	2005	EU436709
HSZ1692-T5	*G. max*	Florida	7	2005	EU436710
HSZ1693-T5	*G. max*	Florida	7	2005	EU436711
HSZ1694-T5	*G. max*	Florida	7	2005	EU436712
HSZ1695-A5	*G. max*	Florida	7	2005	EU436713
HSZ1695-T5	*G. max*	Florida	7	2005	EU436714
HSZ1696-A5	*G. max*	Florida	7	2005	EU436715
HSZ1696-T5	*G. max*	Florida	7	2005	EU436716
HSZ1697-T5	*G. max*	Florida	7	2005	EU436717
HSZ1698-A5	*G. max*	Florida	7	2005	EU436718
HSZ1698-T5	*G. max*	Florida	7	2005	EU436719

(*continued on next page*)

[a] Samples from 1, Cereal Disease Laboratory, St. Paul, MN; 2, J. Markova, Charles University, Czech Republic; 3, Y. Ankister, Institute of Cereal Crops Improvement, Tel Aviv University, Israel; 4, X. Chen, United States Department of Agriculture–Agricultural Research Service (USDA-ARS), Pullman, WA; 5, A. F. Juusten, Department of Crop Protection, Danish Institute of Agricultural Sciences, Denmark, DNA; 6, R. Frederick, USDA-ARS, Foreign Disease–Weed Science Research Unit, Fort Detrick, MD; 7, C. Harmon, Department of Plant Pathology, University of Florida, Gainsville; and 8, N. Glynn, USDA-ARS, Canal Point, FL.

[b] Used only in primer and probe design. DNA was not available.

[c] DNA extracted from infected soybean in BL3 containment facility at Ft. Detrick, MD.

low to record, a blank filter was substituted (NADP blank) and assayed like any other sample. In 2005, nearly all NADP blanks were processed. However, in 2006, only three NADP blanks were processed per week (see Supplementary Tables for details).

Scoring a sample as positive. Assays were considered for scoring only if the positive control was positive and the negative control was negative. If these conditions were not met, the PCR assay was repeated or the data were not included. Rain samples were considered positive for *P. pachyrhizi* if the qPCR assay had a positive C_T value, an S-shaped amplification curve, and produced an ≈250-bp-sized amplicon on a 1.0% agarose gel. The threshold for each reaction was set between 10 and 15 standard deviations above background fluorescence, calculated from cycle 1 through cycle 40. Statistical analysis of the frequency of detecting *P. pachyrhizi* spores over comparable weeks was done using a paired *t* test (Statistix for Windows; Analytical Software, St. Paul, MN).

Assay evaluation. To determine the optimal assay, specificity and sensitivity of each of the three assays was compared. Assay specificity was evaluated using DNA from the same samples used to design the probe when available and from other species (Table 1). Sensitivity of each assay was evaluated using spore suspensions spotted on filters of ≤10, 25, 50, 100, and 500 spores made in sterile deionized water and Tween 20 using a hemacytometer to determine concentration. Each spore suspension was replicated in triplicate and each assay run on the same extraction.

After the optimal assay was determined, single spores were picked using dog hairs (24) to determine whether a single *P. pachyrhizi* spore could be detected and to calibrate the assay (see "Estimating spore load" below). A single spore was picked and examined microscopically before being placed directly into the DNA extraction tube. Each spore was placed directly into the DNA extraction tube and not onto a filter to ensure that a negative result was not due to a spore being trapped on the filter.

Verifying positive samples. To validate that the DNA of a positive reaction was from *P. pachyrhizi*, amplicons were separated by 1.0% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. Furthermore, amplicons from several positive samples with moderate and high spore loads (see below) were cloned and sequenced on an automated DNA sequencer (LI-COR, Lincoln, NE). A minimum of three clones was sequenced for each sample, and the DNA sequence data was assembled and edited with Sequencer (Genecodes, Ann Arbor, MI). If more than one genotype was present, an additional three clones were sequenced. Consensus sequences were compared with other DNA sequences through BLASTN analysis of sequences available on the GenBank website (available online by the National Center for Biotechnology Information). Specific sequence comparisons were made between the amplicons from rain samples and other fungi to pinpoint sequence differences and determine whether the amplicon was derived from *P pachyrhizi* by aligning sequences using MacVector (MacVector, Inc.).

Estimating spore load. To extrapolate the number of spores deposited per square meter over a given week, one spore, two

TABLE 1. (*continued from preceding page*)

Rust species	Host	Origin	Source[a]	Year	GenBank accession no.
HSZ1699	*G. max*	Florida	7	2005	…
HSZ1700	*G. max*	Florida	7	2005	…
HSZ1701-T5	*G. max*	Florida	7	2005	EU436720
HSZ1702-T5	*G. max*	Florida	7	2005	EU436721
HSZ1703-T5	*G. max*	Florida	7	2005	EU436722
HSZ1704-T5	*G. max*	Florida	7	2005	EU436723
P. meibomiae					
Phm	*G. max*	NA	6	2005	…
Brazil 82-1[b]	*Phaseolus lunatus*	Brazil	6	1982	AF333501
Puerto Rico PR[b]	*G. max* and other legumes	Puerto Rico	6	N/A	AF333502
Puccinia andropogonis					
HSZ0218	*Andropogon gerardii*	Wisconsin	1	1998	…
P. coronata					
93MN437	*Avena sativa*	Minnesota	1	1993	AY114290
HSZ0786	*Bromus inermis*	Minnesota	1	2003	…
P. graminis					
78-21-BB463	*Triticum aestivum*	Washington	1	1978	AY114289
56SD37B	*T. aestivum*	South Dakota	1	1956	DQ417383
HSZ0802	*Lolium perenne*	Oregon	1	2003	DQ417384
HSZ0753	*Anthroxanthum* spp.	Czech Republic	2	2003	DQ417386
HSZ1601	*Elymus repens*	Minnesota	1	2006	…
P. hordei					
HSZ0628	*Hordeum murium*	Israel	3	2002	…
P. kuehnii	*Saccharum officinarum*	Florida	8	2007	…
P. melanocephala	*S. officinarum*	Florida	8	2007	…
P. recondita					
91TX9503	*Secale cereale*	Texas	1	1991	DQ417422
HSZ0698	*S. cereale*	Czech Republic	2	2002	DQ417423
ANK9958	*Aegilops ovata*	Israel	3	2002	DQ417424
ANK9974	*A. variabilis*	Israel	3	2003	AY187088
P. striiformis					
PST78	*T. aestivum*	Washington	4	2001	DQ417396
22/99	*T. aestivum*	Denmark	5	1999	DQ417397
52/99[b]	*T. aestivum*	Denmark	5	1999	DQ417404
HSZ0722	*T. aestivum*	Texas	1	2003	DQ417405
P. triticina					
HSZ0741	*T. aestivum*	Czech Republic	1	2002	DQ417409
98EGY151C	*T. aestivum*	Egypt	1	1998	DQ417419
ARG-509	*T. aestivum*	Argentina	1	2005	…
BRA-25.1	*T. aestivum*	Brazil	1	2004	…
PER-94-28	*T. aestivum*	Peru	1	1994	…
URG-27.2	*T. aestivum*	Uruguay	1	2004	…

spores, and five spores were assayed following the nested qPCR protocol. Individual spores were picked using dog hair as described above. Placing spores in tubes directly, rather than on filters, was done to ensure that a known number of spores were assayed. Logarithmic and power curves (Fig. 2A and B, respectively) were fitted to the average C_T value of each spore number (n = 11, n = 5, and n = 4 for one, two, and five spores, respectively) using Delta Graph 5.6 (Red Rock Software, Salt Lake City, UT). Both models were used to estimate spore loads from C_T values (Fig. 2C). Some C_T values of the two-spore DNA extracts were not included in the average because they were noticeably higher than others, matching C_T values from one spore, and most likely resulted from DNA not being obtained from both spores. It was not feasible to run standard curves for each assay because the DNA extracts from one and two spores were unstable over just a few days time, even when stored at –20 or –80°C, and practically could not be made new each day. Therefore, the fitted curves were used to estimate spore load for all samples. When model estimates differed by more than a few spores per square meter, a range was given using both equations. Otherwise, values obtained from the logarithmic equation were used.

Genotypic variation. The primary objective in sequencing a subset of rain samples testing positive for *P. pachyrhizi* was to verify the nested qPCR assay. However, an amplicon (ITS1) sequence of *P. pachyrhizi* from rain samples was also used to estimate genotypic variation within the rain collected over the soybean-growing regions of the continental United States. Additionally, infected soybean leaves were collected at 20 points in a single sentinel plot in Marion County, FL, by Dr. Carrie Lapaire Harmon (University of Florida) (Table 1) for the purpose of providing a positive DNA control for the nested qPCR assay. These samples were also used to estimate the genetic variation of the ITS1 region within a single field. DNA was extracted from infected leaves from the sentinel plot using the same methods as for the rain filters. Nuclear rDNA was PCR amplified in a 50-µl reaction mixture as described by Anikster et al. (1). DNA sequencing reactions were preformed using a Thermo Sequenase Primer Cycle sequencing kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and analyzed on an automated DNA sequencer (LI-COR). A minimum of three clones was sequenced for each sample and the DNA sequence data was assembled and edited with Sequencer (Genecodes). Nucleotide sequence data have been submitted to GenBank, with the accession numbers listed in Tables 1 and 2. The variation in ITS1 sequence among the 13 sequences from GenBank, 18 of the 20 DNA samples of infected soybean leaves from the Florida sentinel plot, and a subset of positive rain samples in 2005 and 2006 were aligned using MacVector (version 9.0; MacVector Inc.). Polymorphic sites were considered legitimate if they occurred in more than two samples.

Disease development with LDD. Positive samples were plotted using GPS coordinates of the individual NADP/NTN sites in ArcGIS 9.1 (ESRI, Redlands, CA). To analyze incidence of LDD, samples testing positive for *P. pachyrhizi* were correlated with jumps in counties reporting SBR in the field using ArcGIS to measure distances between counties reporting SBR. Four individual reports of disease greater than 100 km from the next nearest county or parish report were mapped beginning 20 August 2006. Contour maps were constructed using Adobe Photoshop CS 8.0 (Adobe Systems Incorporated, San Jose, CA).

RESULTS

Assay evaluation. Specificity of the nested assay was evaluated using DNA from a variety of rust fungal species (Table 1). In all cases, the assay detected only *P. pachyrhizi*. Specificity of the two non-nested assays has been previously shown (2,8), with the modified ITS1rustR3d primer showing the same specificity as ITS1rustR3c (2). Specificity of the ITS1PhpFAM1 TaqMan probe was further evaluated by BLASTN analysis. In addition to matching to *P. pachyrhizi*, the next closest match to the TaqMan probe was to a protozoan (*Tetrahymena thermophila*) with only 63% similarity.

Spore suspensions of ≤10, 25, 50, 100, and 500 spores were created to evaluate all three assays. Both single-round assays performed equally well down to 25 spores/filter, but neither detected *P. pachyrhizi* DNA as consistently as the nested assay at the ≤10 spores/filter concentration. The nested assay detected *P. pachyrhizi* in every case where the two single-round assays did not. The results also indicated that the protocol for removing spores from filters was effective at low spore concentrations.

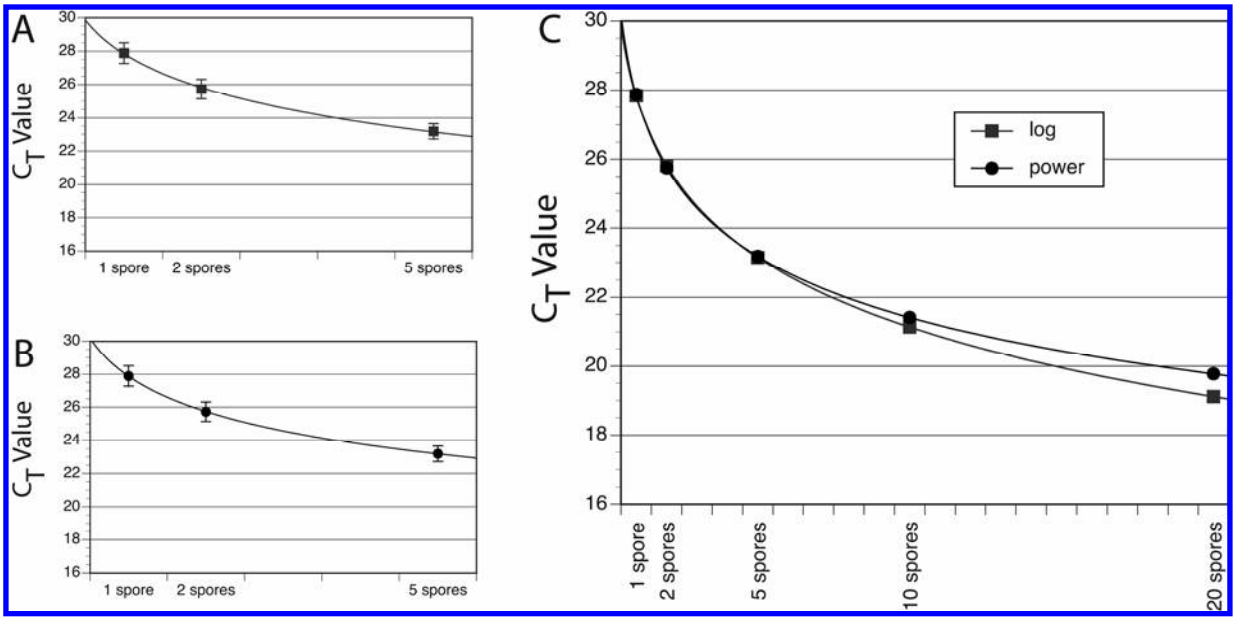

Fig. 2. Standard curves used to estimate the numbers of spores (spore load) detected in an assay. Two equations were used to estimate spore load: **A,** Logarithmic equation ($Y = -2.911 \times \mathrm{Ln}(x) + 2.78$) and **B,** power equation ($Y = \exp(3.328) \times X^{0.115}$). Both curves were fitted to average threshold cycle (C_T) values obtained by assaying one (n = 11), two (n = 5), and five (n = 4) *Phakopsora pachyrhizi* spores using the nested real-time polymerase chain reaction assay in this study. Standard error bars are shown. **C,** Estimation model where C_T values were used to estimate the number of spores from rain collected during the 1-week collection period. In total, 95% of all positive samples had ≤5 spores.

Because the two single-round assays did not always detect ≤10 spores/filter, only the nested assay was evaluated for *P. pachyrhizi* spores picked with dog hair. In this experiment, single spores were detected 67% of the time with the nested assay.

Picking single spores with dog hair was also used to calibrate the assay to estimate the number of spores detected in rain samples. The logarithmic equation (Fig. 2A) was used to estimate the spore load for spore suspensions of ≤10, originally used to evaluate all three assays. The results (n = 30, mean = 4.0, standard deviation = 3.1, minimum = 0.9, maximum = 15.5) suggest that placing spores directly in tubes rather than on filters was appropriate for calibrating the assay.

Verifying positive samples. In all, 1,461 rain samples were collected in 2005 and 2,145 samples were collected in 2006. All samples with a positive C_T value were analyzed by gel electrophoresis. Amplicons of the expected size (≈250 bp) were observed on 1.0% agarose gels for all samples testing positive with the *P. pachyrhizi* qPCR assay and with an S-shape amplification curve. As expected, samples with higher spore loads (lower C_T values) had amplicons with brighter fluorescence. Occasionally, a sample with a low spore load score did not result in a visible amplicon on the accompanying agarose gel and was scored as negative.

In total, 6 and 31 randomly chosen rain samples that had >20 spores/m^2 were sequenced in 2005 and 2006, respectively. All 37 DNA samples were found to be from *P. pachyrhizi* based on sequence alignments and BLASTN results.

Detection frequency. There was a significant increase in the number of rain samples testing positive for *P. pachyrhizi* per week in 2006 compared with 2005 over the same time frame (paired t = 4.45, DF = 15, P = 0.0005), with no significant difference in the number of rain samples assayed (paired t = 0.97, DF = 15, P = 0.35). Of the 1,461 rain samples assayed in 2005 from the 121 NADP sites, 76 were positive for *P. pachyrhizi*. In 2006, over the same time period from 9 May through August, 268 of the 1,509 rain samples assayed tested positive. As a percentage, 5.2% of the assays showed positive results for *P. pachyrhizi* in 2005, and 17.8% in 2006 over the same time period.

A bimodal pattern of detecting positive rain samples was found in both years. The number of positive samples increased in May, decreased in mid-June to mid-July, and increased again in late July (Fig. 3). In 2005, the number of positive samples began to decline in mid-August. In 2006, the number of samples testing positive for *P. pachyrhizi* was still high at the end of August and did not decline until mid-September. From the beginning of September through 17 October 2006, 636 rain samples were assayed for *P. pachyrhizi.* The total number of rain samples assayed in 2006 increased to 2,145 and the total number of samples testing positive for *P. pachyrhizi* increased to 327 samples (15.2%). The bimodal pattern of detecting positive samples was not the result of matching increases and decreases to the frequency of rainfall. Although, in 2005 there was a decrease in the number of sites with rain in mid-June, there was no difference in the number of sites with measurable rain in any other month and no pattern at all in 2006 (data not shown).

Detection distribution. *P. pachyrhizi* was detected across the U.S. soybean-growing region in both 2005 and 2006 (Fig. 4A and B, respectively). Also common between years was a band of low-frequency detection of *P. pachyrhizi* spores in rain from South Carolina directly west to northern Texas. However, in 2005, the majority of the rain samples testing positive for *P. pachyrhizi* were in the southern United States, south of the Ohio River. In 2006, there was a high frequency of positives along the Gulf Coast and Texas but also an increase in the frequency of samples testing positive for *P. pachyrhizi* north and northwest of the Ohio River compared with 2005. The increase in frequency of detection and higher spore loads in the upper Midwest corresponds to the change in disease reports between years (Fig. 4A and B) (Integrated Pest Management–Pest Information Platform for Extension and Education [IPM PIPE] website).

Each year in the first 2 weeks of the study, spore deposition in rain was primarily in the southern United States. In 2005, the pattern was observed by detection alone whereas, in 2006, relatively higher spore loads were detected in the southern United

TABLE 2. List of genotypes found from a subset of rain samples with *Phakopsora pachyrhizi* spores

Date[a]	Site[b]	Genotype[c]	Accession no.
28 June 2005	WI28	T4	EU584435
		T5	EU584436
5 July 2005	MO05	T5	EU584437
26 July 2005	MN27	T4	EU584438
		T5	EU584439
9 August 2005	ND08	T5	EU584440
23 August 2005	IN41	T5	EU584441
23 August 2005	SD99	A6	EU584442
16 May 2006	GA99	A5	EU584443
		T4	EU584444
		T5	EU584445
	LA12	T5	EU584446
	VA24	T5	EU584447
23 May 2006	FL99	A5	EU584448
		T5	EU584449
	IL63	A5	EU584450
		T5	EU584451
	KY03	T5	EU584452
	MI51	A5	EU584453
		T4	EU584454
		T5	EU584455
	NC41	A5	EU584456
		C5	EU584457
		T5	EU584458
30 May 2006	KS07	A5	EU584459
		T5	EU584460
	TN00	A5	EU584461
		T5	EU584462
	VA13	A5	EU584463
		T5	EU584464
6 June 2006	GA99	A5	EU584465
		T5	EU584466
	KS07	T5	EU584467
		T6	EU584468
	MN01	T5	EU584469
	OH09	T5	EU584470
	SD08	T4	EU584471
		T5	EU584472
	TN04	A5	EU584473
		T5	EU584474
	TX22	T5	EU584475
13 June 2006	MN23	T5	EU584476
	NC03	A5	EU584477
	OH09	T5	EU584478
20 June 2006	IL18	A5	EU584479
	KS32	T4	EU584480
		T5	EU584481
27 June 2006	MO03	A5	EU584482
		C5	EU584483
		T4	EU584484
		T5	EU584485
	MO43	T5	EU584486
	SC05	T5	EU584487
25 July 2006	PA15	T5	EU584488
	TX03	T4	EU584489
		T5	EU584490
1 August 2006	AL99	T5	EU584491
	IA23	A5	EU584492
		T5	EU584493
	MI52	T4	EU584494
		T5	EU584495

[a] Date precipitation was collected. Precipitation was collected at weekly intervals.
[b] National Atmospheric Deposition/National Trends Network sites.
[c] The letter corresponds to the nucleotide of the single nucleotide polymorphism followed by the number of TA simple sequence repeats (Fig. 5).

States during the same time frame (Table 3). From 10 to 17 May 2005, rain samples that tested positive for *P. pachyrhizi* were found in South Carolina and Texas at relatively low spore load levels. During the second week, 17–24 May 2005, low spore load samples were found in South Carolina and twice in Tennessee. For the week of 9–16 May 2006, moderate spore loads were detected in Georgia, Louisiana, and Virginia, with low spore loads detected in Kansas, Kentucky, and Pennsylvania. During the second week in 2006 (16–23 May), moderate spore loads were detected in Florida, Illinois, Kentucky, Michigan, and North Carolina. Low spores loads for the second week in 2006 were found in Illinois, Indiana, Minnesota, Missouri, twice in North Carolina, and in Ohio (Table 3). Overall, *P. pachyrhizi* spores were detected more frequently and at higher levels in 2006 than in 2005 (Table 4).

To observe the relationship of LDD of spores and disease incidence, reports of disease greater than 100 km from the next nearest county or parish report were mapped beginning 20 August 2006 (Fig. 4C). The distribution of positive samples with >20 spores/m^2 in August and September showed two generalized pathways for LDD, one along the east coast and the other up along the Mississippi River toward Iowa, Illinois, Indiana, and western Ohio. These two pathways correspond to the field reports reported by PIPE of SBR in the field in September and October. SBR was first reported in Kentucky on 11 October 2006 (Fig. 4C). Rain collected on 19 and 26 September tested positive for *P.*

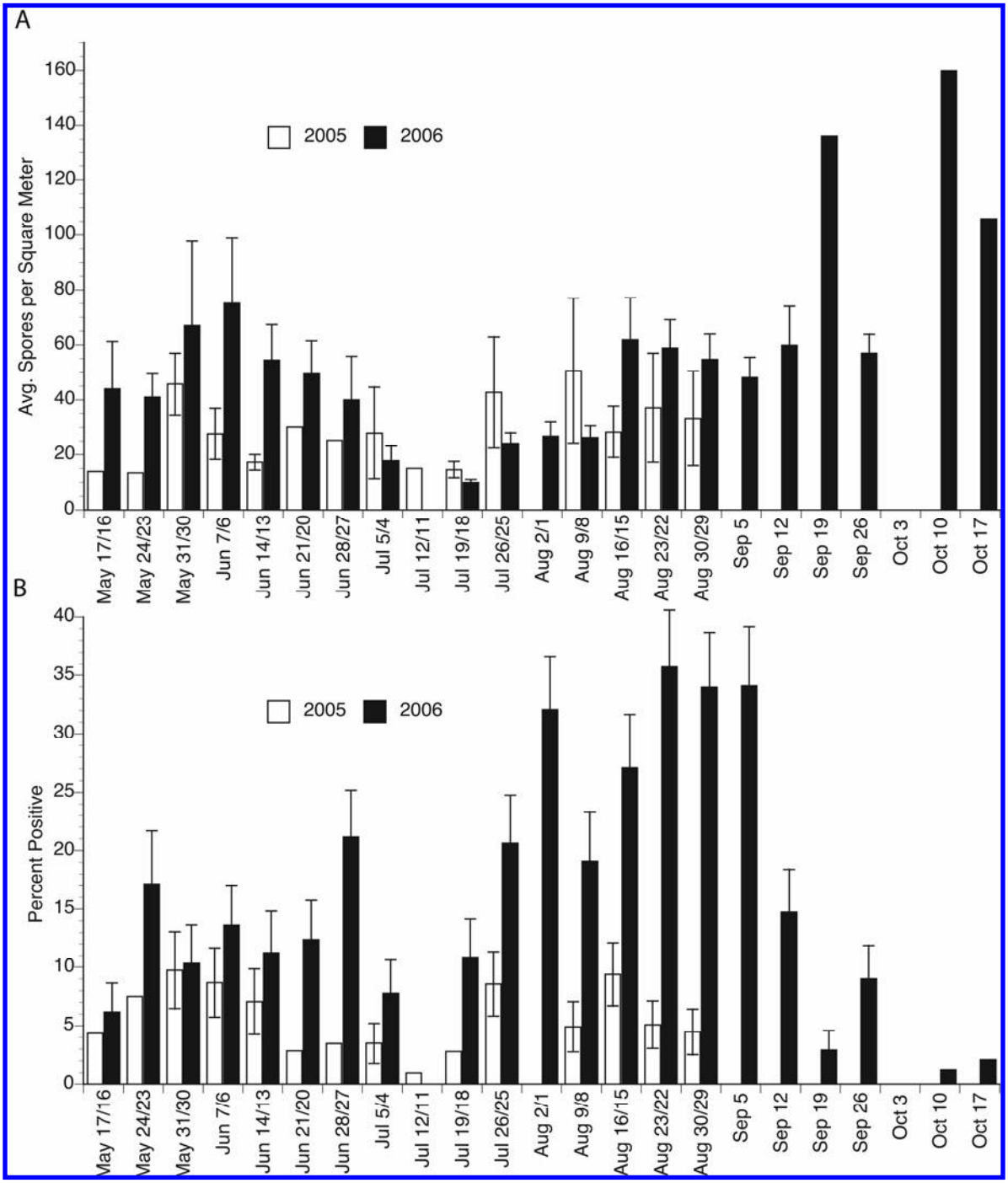

Fig. 3. A, Average number of spores per square meter and **B.** frequency of rain samples testing positive for *Phakopsora pachyrhizi*. Rain was collected weekly at 121 National Atmospheric Deposition Program/National Trends Network sites in 2005 and 110 sites in 2006. Standard error bars are shown for samples with >3 data points only. Dates in the x-axis represent 2005/2006, respectively.

pachyrhizi at an NADP site (KY99) in the same county as the SBR report. The sample on 19 September was the third highest spore load recorded that year (317 to 361 spores/m^2, log and power equations, respectively), while the sample collected on 26 September had ≈78 spores/m^2.

To examine possible differences between the two generalized pathways, NADP sites were divided into eastern and western groups along 85°W longitude to make two equal groups (Fig. 4C). There was an easterly shift in the frequency of spore load positives >20 spores/m^2 from August through September 2006. In the 5 weeks of August, four of five showed higher frequency of detection of *P. pachyrhizi* spores in the western half of the study compared with the eastern half. In September, the percentage of sites in the eastern half of the study were always higher compared with sites in the western half of the study (Fig. 4D).

Genotypic variation. To estimate genotypic variation among *P. pachyrhizi* found in rain, two genotypic markers were observed from 98 sequences: 24 sequences from 18 infected soybean leaf samples from the Florida sentinel plot, 13 sequences from GenBank, 8 sequences from 6 rain samples in 2005, and 53 sequences from 31 rain samples in 2006. A single nucleotide polymorphism and simple sequence repeats were used to create a genotype designation and resulted in six different genotypes for all sequences (Fig. 5).

Two genotypes were found in the single sentinel plot in Florida. In all, 61% (11/18) of the samples were of the T5 genotype, 1 of 18 samples had the A5 genotype alone, and the remaining 6 samples (6/18) had both genotypes. These two genotypes were also found in sequence available in GenBank (Table 1) and were the most common genotypes found in the rain samples (Fig. 5).

DNA was sequenced from 6 positive rain samples in 2005 and 31 in 2006. All were found to be from *P. pachyrhizi*, confirming the results of the nested qPCR assay. Two predominant genotypes, A5 and T5, were found across the study area. However, four other genotypes were also observed. Two of these genotypes, A6 and T6, correspond to sequences found in GenBank, while genotypes T4 and C5 were unique (Fig. 5). Of the six samples testing positive for *P. pachyrhizi* in 2005, three genotypes were found, and two of the six samples had two genotypes. In 2006, 18 of the 31 samples had more than one genotype (Table 2). Of those samples with more than one genotype, 15 had two genotypes, 3 had three genotypes, and 1 sample had four genotypes. The sample with four genotypes was found in Missouri the week ending 27 June 2006.

DISCUSSION

The first objective of this study was to develop a qPCR assay that would be sensitive and accurate enough to detect a single *P.*

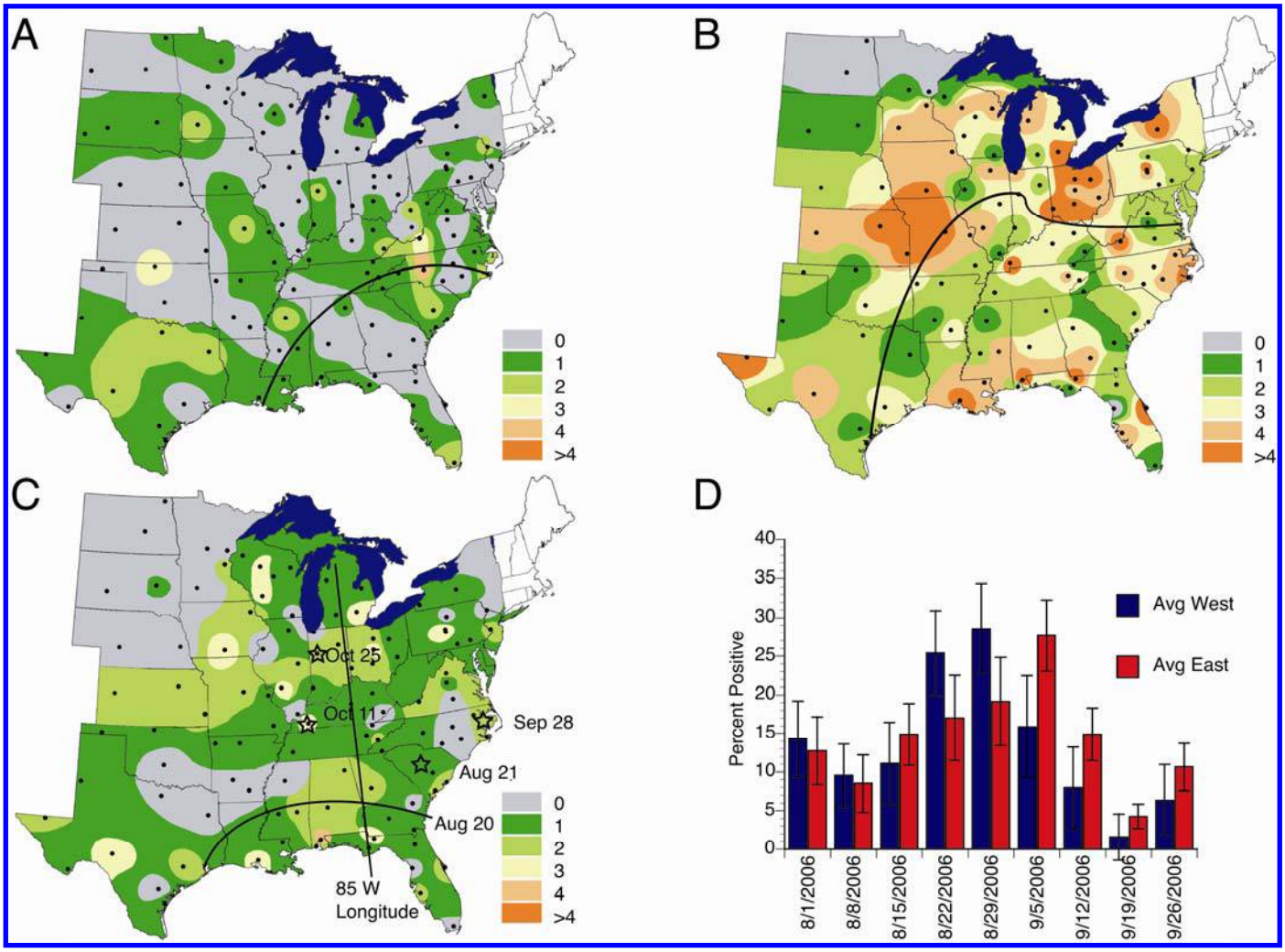

Fig. 4. Geographic distribution and frequency of rain samples positive for *Phakopsora pachyrhizi* collected **A,** from 10 May through 30 August 2005 and **B,** from 9 May through 17 October 2006. Northern boundary of disease reports from Pest Information Platform for Extension and Education website for each year through October are indicated by an arced line. Frequency of positive samples is indicated by the color legends adjacent to the maps. **C,** Distribution of spore loads >20 spores/m^2 for August and September 2006. Locations and dates of where disease reports were >100 km from previously reported locations are shown (star). The arced line indicates the northern limit of disease reports as of 20 August 2006. **D,** Percentage of filters testing positive with moderate and high spore loads were averaged by sites west and east of the 85th longitude to illustrate an eastwardly shift in spore deposition in August and September. Standard error bars are shown.

pachyrhizi spore. In laboratory simulations, the assay detected a single *P. pachyrhizi* spore in roughly two out of three instances, and ≥2 spores 100% of the time, demonstrating the sensitivity of the assay. The implication then was that the assay could be used to detect the primary deposition of inoculum of SBR. The strength of the nested assay was that it was based on two levels of specificity in the primers and in a TaqMan probe, with the shape of the amplification curve and amplicon size used to confirm positive C_T values. To further evaluate accuracy, qPCR amplicons from 37 rain samples, 6 from 2005 and 31 from 2006, with a positive assay score were sequenced. All the rain samples were *P. pachyrhizi* based on sequence alignment, proving the accuracy of the assay. These results indicate that the assay developed in this study is useful in detecting spores deposited in rain, and that it was sensitive enough to detect the first deposition of spores into a given area due to LDD, as well as subsequent local spore transport.

With a reliable assay in hand, the second objective of this study was to monitor rainfall for *P. pachyrhizi* spores and assess deposition patterns over the soybean-growing areas of the central United States. In this study, >1,400 rain samples were assayed for *P. pachyrhizi* in 2005 and >2,100 in 2006 at more than 100 NADP/NTN locations in 29 and 28 states, respectively, each year (Fig. 3). As expected, based on the fact that SBR was first observed in the continental United States in the fall of 2004 (26), fewer rain samples were detected with *P. pachyrhizi* spores in 2005 than in 2006. The roughly threefold increase in the percentage of rain samples with *P. pachyrhizi* spores indicates an increase in incidence or severity of infected hosts in the inoculum source regions. Furthermore, the bimodal pattern found both years, with an increased frequency of detection first in late May

TABLE 4. Summary of spore load of weekly rain collections in 2005 and 2006[a]

Year	Low[b]	Moderate[c]	High[d]	Very high[e]
2005				
Number	49	19	8	0
Percent	64	29	7	0
2006				
Number	118	155	43	11
Percent	36	48	13	3

[a] All spore load estimates were done using the logarithmic equation $Y = -2.911 \times \mathrm{Ln}(x) + 2.78$.
[b] Low spore load corresponded to <20 spores/m^2.
[c] Moderate spore load corresponded to 20 to 80 spores/m^2.
[d] High spore load corresponded to 81 to 200 spores/m^2.
[e] Very high spore load corresponded to >201 spores/m^2.

TABLE 3. List of positive rain samples by state and date

Date[a]	No.[b]	States where *Phakopsora pachyrhizi* spores were detected in rain[c]
2005		
May 17	2	SC, TX
May 24	3	SC, TN (2)
May 31	8	*TX*, *NC*, TX (2), VI, MN, TX (2)
June 7	8	IA, MO, NC, AL, AR, FL (3)
June 14	6	NC, KY, MO, OK, WV (2)
June 21	2	IN, OK
June 28	3	WI, NY, VA
July 5	4	MO, TN, TX, VA
July 12	1	NC
July 19	3	SC, FL, SC
July 26	9	*MN, SD*, PA, KY, MN, MO, PA (2), VA
August 2	0	
August 9	5	*ND*, NC (2), MS, NC
August 16	11	*MI*, KY, MN, NC, KY, MS, NC, OH, OK, WV, TX
August 23	6	*SD*, IN, AL (2), FL, SD,
August 30	5	*PA*, VA, IN, TX (2)
2006		
May 16	6	*LA, VA*, GA, KS, KY, PA
May 23	12	*KY*, FL, IL (2), IN, OH, MI, MN, NC (2), MO, NC
May 30	9	***VA***, *TN*, KS, KY, MI, MN, MI, NY (2)
June 6	14	***OH***, *PA, SD*, GA, KS, MN, OH (2), OK, TN, TX, WI, FL, MI
June 13	9	*KY, MN, NC, OH*, NC, NY, TX, OH, NC
June 20	12	*IL, KY*, FL, GA (2), KS (2), MS, NC, NE, GA, LA
June 27	22	***SC***, *MO*, IA, MO, OH, PA, NY, TX, AL, AR (3), FL, MO, NC (4), OH (4)
July 4	7	PA, IN, KS, MI, PA, SC, WI
July 11	0	
July 18	10	AR, IN, KS, KY (2), MN (2), MO, OH, OK
July 25	20	IN, NE, OH (2), OK, PA (2), TN, TX (2), WI, FL (2), IA, MI, PA (2), SC, TX, VA
August 1	34	*AL, FL,WI,* AL (2), FL, IA, KS, MI, MS, NC, OK, PA, TX, VA, AR(2), FL, GA, IA, IL, KS, LA, MO, MS, NC (2), NE (2), NY, PA, TN, TX, WI
August 8	17	FL, IN, KS, NJ, OH (2), TX (2), WI, IA, OH, TX (3), VA, WI (2)
August 15	26	***IL, TN***, *IA, IL, NY, PA, SC, TX*, AL, FL, IA, IL, NC (2), OH, IL, IN, MO, NC (2), NY, OH (3), TN (2)
August 22	35	***IN, OH, VA***, *AL, KS, MO, NY, TX (2),* AR, FL, GA, IN, KS (2), KY, MI (3), MN, NC (2), TX, WI, AR, FL (2), IL, LA (2), MS (2), NC, OH, PA,
August 29	35	***TN***, *OH, TN, TX, WI,* AL, FL, IL, IN (3), KY (2), LA (2), MI (3), MN, MO (3), MS, NJ, OH (3), OK, WI (2), AL, FL, KY, MN, WI
September 5	30	***PA***, *AL, IL, PA, VA,* FL, GA, IA, KS, LA, MS, OH, PA (2), SC (2), SD, TX, VA, WI (2), FL, NC (3), NE, OH, TN, TX, VA
September 12	14	*AL, GA, VA,* AL, AR, FL, GA, IA, LA, NC, OH, SC, OH, OK
September 19	3	***KY***, FL, MI
September 26	9	*PA*, AL, IL, KY, OH, PA (2), SC, WI
October 3	0	
October 10	1	*NC*
October 17	2	*IL, TX*

[a] Date precipitation was removed from the National Atmospheric Deposition collector. Precipitation was collected each week.
[b] Number of rain samples testing positive for a given week.
[c] List of states with positive samples separated by spore load. ***Bold italic***, >201 spores/m^2; *italic*, 81 to 200 spores/m^2; underline, 20 to 80 spores/m^2; and regular text, <20 spores/m^2 based on the log model. Parentheses indicate multiple sites within a state with the same spore load range.

and then a larger increase in the beginning of August, suggests a possible shift of the primary inoculum sources from, primarily, the weedy legume kudzu in the spring to soybean. The larger increase in August likely reflects the increase in soybean availability and, perhaps, susceptibility. The roughly threefold increase in frequency of detecting *P. pachyrhizi* spores in rain in 2006 mirrored the increase, also roughly threefold, in the number of counties or parishes reporting detection of SBR in the field (PIPE website). The field reports do support the findings of this study that more inoculum was being distributed in 2006 compared with 2005 over a larger area and for a longer period of time.

The results of this study showed that frequency of deposition and relatively higher spore numbers of *P. pachyrhizi* spores were primarily in the southern United States in May and early June both years, but moved further north sooner in 2006 relative to 2005. These findings are consistent with models by Pivonia and Yang (20) that suggested that spore sources would be restricted to the most southern parts of Florida and Texas and also Mexico in the winter and spring months. Specific NADP/NTN sites positive for *P. pachyrhizi* in this study have been used in HYSPLIT back trajectory models and showed that the source of spores matched those predicted (13). However, *P. pachyrhizi* spore deposition was not entirely restricted to the southern United States. By the end of May in 2005 and more so in 2006, *P. pachyrhizi* spores were detected as far north as Minnesota, Illinois, Indiana, Michigan, Ohio, and Pennsylvania, but at lower frequency and spore numbers. Rowell and Romig (25) found *P. graminis* and *P. triticina* in rain samples in Minnesota in early May in 1963 and 1964.

There was a general trend of *P. pachyrhizi* spores being deposited further north and west in 2006 compared with 2005. In 2005, most of the sites where *P. pachyrhizi* spores were detected were south of the Ohio River. However, *P. pachyrhizi* spores were also detected with relatively high frequency in Texas and Oklahoma both years (Fig. 3A and B). These findings suggest that inoculum sources were not restricted to the southeastern United States. To evaluate spore deposition in the central plain states, with inoculum sources potentially in Texas or Mexico, frequency of detection at sites east and west of 85°W longitude were averaged from August through September 2006 (Fig. 3C). The general pattern that *P. pachyrhizi* spores are detected in the western half of the study area roughly a week before being detected in the east suggests a more westerly and perhaps earlier-maturing source of spores than Florida or its surrounding states. The movement of spore deposition from west to east, following the general storm patterns of the United States at this time of year, further validates the findings of this study.

For LDD of spores to be relevant to disease development, spores must be viable. This study did not test for spore viability, and could not as a DNA assay. The detrimental effects of ultraviolet radiation on spore survival have been documented (18,21) and, along with the need for a virulent pathogen, susceptible host, and favorable environmental conditions, using these data alone as a predictor for disease is risky. However, indirect evidence for LDD of viable spores in this study was the relatively long distances between isolated diseased fields in South and North Carolina, western Kentucky, and west-central Indiana in the fall of 2006. These areas did correspond to areas with relatively high and moderate spore deposition 3 to 4 weeks prior to disease development, suggesting LDD of inoculum. A distinction should be made with respect to primary infections and first detection in the field, which may differ greatly.

LDD of viable fungi has been reported by multiple authors (6,16,21,22,25,27). The literature suggests that viable spores are transported considerable distances and deposited in rain. However, there are other confounding factors beyond host susceptibility and environmental conditions that relate to disease development. Independently, Roelfs, while working in Kansas, and Rowell, in Minnesota (A. P. Roelfs, *personal communication*), found that 1 *Puccinia graminis* spore in 10 viable spores would cause infection on susceptible wheat but 1 *P. triticina* spore in 3 viable spores would cause infection, with other factors being equal. Therefore, the biology of *Phakopsora pachyrhizi* regarding infection efficiency will need to be taken into account in models predicting disease development beyond simple spore deposition or spore viability.

The primary goal of sequencing qPCR products was to verify the assay results. However, some insight could be made into the population structure of *P. pachyrhizi* in the United States based on the genotypic variation found in the rain samples over the 2-year period of this study, along with that found in a single sentinel plot. A more comprehensive study of genotypes may reveal temporal and geographic patterns that cannot be ascertained by the limited sampling in this study. However, comparing ITS1 sequences from the rain samples and the sentinel plot did show some patterns. The two genotypes found in the sentinel plot, T5 and A5, were the same two predominant genotypes found in the rain samples, suggesting that there is a predominant pathogen population across the United States and, perhaps, in the original source or sources. The results from the sentinel plots, where one-third of the single-leaf samples had both genotypes, suggests that local populations are also genotypically diverse. The dominant genotype in the sentinel plot was also predominant in the rain

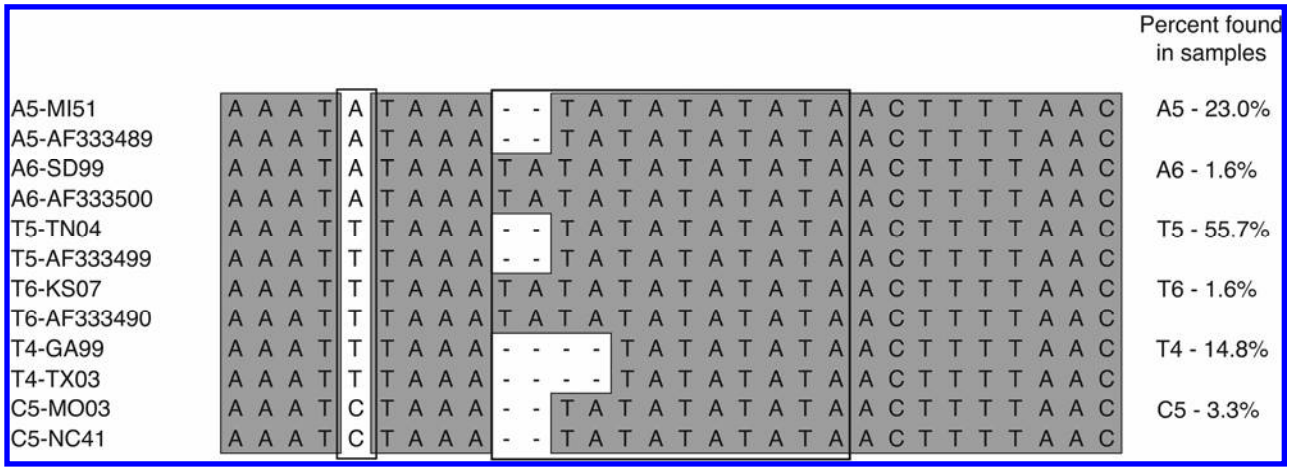

Fig. 5. Nucleotide sequence consensus alignment of the 3′ end of the internal transcribed spacer 1 (ITS1) region between samples from this study (genotype followed by site locations) and downloaded sequence from the National Center for Biotechnology Information (with accession numbers). Percentage of genotypes found in rain samples in 2005 and 2006 are to the right of the sequences. Genotypes T4 and C5 are unique genotypes not previously identified. The genotype codes were generated from the single nucleotide polymorphism substitution and the variation in the number of TA repeats. Both sites are delimited by boxes.

samples across the study area over both years and among sequences found in GenBank from isolates collected worldwide. Also, two unique genotypes, T4 and C5, were found that are not found in GenBank. Therefore, genotypic diversity within the population of *P. pachyrhizi* across the United States may be of a predominant genotype but with lower levels of other genotypes mixed in the population. The results of this study do not show a geographic pattern to any particular genotype, noting that sampling for genotypic diversity was not the objective of the study. However, the diversity of genotypes found in this study suggests that either the initial introduction of *P. pachyrhizi* was genotypically diverse, the diversity is a result of multiple introductions of different genotypes, or both.

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